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W.R. GRACE & COMPANY

Contractor

LITTON BIONETICS INC

Document Title

MUTAGENICITY EVALUATION OF LIQUID POLYMER 11664-97 IN THE AMES SALMONELLA/MICROSOME PLATE TEST (FINAL REPORT) WITH COVER LETTER DATED 011691

Chemical Category

4,4-DIPHENYLMETHANE DIISOCYANATE (101-68-8)

GRACE 86-9100006 48

Joseph W. Raksis, Vice President
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91 JAN 24 AM 9:44

January 16, 1

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Attn: Health and Safety Reporting Rule (Notification/Reporting)

Please find attached 8(d) health and safety reports for mixtures processed containing toluene diisocyanate (CAS #26471-62-5), 4,4-Diphenylmethane diisocyanate (CAS #101-68-8) and 1,6-Diisocyanatohexane (CAS #822-06-0). Grace is submitting these reports for late filing since their submittal may have been subject to the isocyanates 10-year call-in of June 1, 1987.

We have reason to believe that some of these reports may have previously been submitted to EPA as attachments to PMN submissions. However, Grace is filing them as a precautionary measure to insure EPA's receipt.

These reports are being submitted for:

W. R. Grace & Co.-Conn.
Washington Research Center
7379 Route 32
Columbia, MD 21044

Sincerely,

J. W. Raksis

A:\JR91-013/lw

Attachments - 20

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GENETICS ASSAY NO. 5919

CONTAINS NO CBI

LBI SAFETY NO. 7237

4,4-Diphenylmethane diisocyanate
101-68-8

MUTAGENICITY EVALUATION OF

LIQUID POLYMER 11664-97

IN THE

AMES SALMONELLA/MICROSOME PLATE TEST

FINAL REPORT

SUBMITTED TO:

W.R. GRACE AND CO.
7379 ROUTE 32
COLUMBIA, MD 21044

SUBMITTED BY:

LITTON BIONETICS, INC.
5516 NICHOLSON LANE
KENSINGTON, MARYLAND 207953

LBI PROJECT NO. 20988

REPORT DATE: NOVEMBER, 1981

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Litton BIONETICS

86910000648

PREFACE

This report contains a summary of the data compiled during the evaluation of the test compound. The report is organized to present the results in a concise and easily interpretable manner. The first part contains Items I-IX. Items I-IV provide sponsor and test article identification information, type of assay, and the protocol reference number. Item V provides the initiation and completion dates of the study. Item VI identifies the supervisory personnel. Item VII indicates the tables and/or figures containing the test results. The interpretation of the results is in Item VIII. Item IX provides the conclusion and evaluation.

The second part of the report describes the study design, which includes the materials and procedures employed in conducting the assay. This part of the report also contains evaluation criteria used by the study director, and any appendices.

All test and control results presented in this report are supported by raw data which are permanently maintained in the files of the Department of Molecular Toxicology or in the archives of Litton Bionetics, Inc., 5516 Nicholson Lane, Kensington, Maryland 20895.

Copies of the raw data will be supplied to the sponsor upon request.

The described study was performed in accordance with Good Laboratory Practice regulations except if noted to the contrary. To the best of the signer's knowledge there were no significant deviations from the Good Laboratory Practice regulations which affected the quality or integrity of the study.

I. SPONSOR: W.R. Grace and Co.

II. MATERIAL (TEST COMPOUND): GENETICS ASSAY NUMBER: 5919

A. Identification: Liquid Polymer 11664-97

B. Date Received: September 24, 1981

C. Physical Description: Viscous, yellow liquid

III. TYPE OF ASSAY: Ames Salmonella/microsome Mutagenesis Assay

IV. PROTOCOL NUMBER: 401

V. STUDY DATES:

A. Initiation: October 9, 1981

B. Completion: October 29, 1981

VI. STUDY DIRECTOR: D.R. Jagannath, Ph.D.

VII. RESULTS:

The results of this assay are presented in Tables 1 and 2.

VIII. INTERPRETATION OF RESULTS:

The test compound was examined for mutagenic activity in a series of in vitro microbial assays employing Salmonella indicator organisms. The compound was tested directly and in the presence of liver microsomal enzyme preparations from Aroclor-induced rats.

A negative control consisting of the solvent used for preparing the stock solutions and subsequent dilutions of the test material and specific positive compounds were also assayed concurrently with the test material. The negative control data was used as the base for evaluating the results obtained with the test material.

DOSE RANGE

A preliminary toxicity study conducted on the test material at 14 doses of 1.22 µg to 10,000.0 µg per plate using the strain TA-100, did not exhibit significant toxicity at any of the doses (Table 1). As such, the mutagenicity assays were conducted at 8 doses of 1.0 µg to 10,000.0 µg per plate.

VIII. INTERPRETATION OF RESULTS: (continued)

The results of the tests conducted on the test material in the absence of a metabolic activation system were negative. The tests with TA-1538 and TA-100 were repeated because of the low number of revertants observed at various test doses in the initial assay. The repeat tests were negative.

The results of the tests conducted on the test material in the presence of a rat liver activation system were negative. The test with TA-98 was repeated since there were no revertants on the solvent control plates in the initial assay attributed to a technical error in plating the cells. The repeat test was negative. The tests with TA-100 was repeated because of the low number of revertants observed at various test doses in the initial assay. The repeat test was negative.

The test with TA-1535 was repeated in the activation and nonactivation assays because of the failure of the positive control to revert the strain in the initial assay. The repeat tests were negative.

IX. CONCLUSIONS:

The test material, Liquid polymer 11664-97, did not exhibit genetic activity in any of the assays conducted in this evaluation and was considered not mutagenic under these test conditions according to our evaluation criteria.

Submitted by:

Study Director

D.R. Jagannath 11/19/81

D.R. Jagannath, Ph.D. Date
Section Chief
Submammalian Genetics
Department of
Molecular Toxicology

Reviewed by:

David J. Brusick 11/19/81

David J. Brusick, Ph.D. Date
Director
Department of
Molecular Toxicology

TABLE 1

TOXICITY TEST WITH TA-100

SPONSOR: W.R. Grace and Co.

COMPOUND CODE: Liquid Polymer 11664-97

ASSAY NO.: 5919

SOLVENT: DMSO

DATE INITIATED: 10/9/81 DATE COMPLETED: 10/12/81

TEST COMPOUND UG/PLATE	NUMBER OF COLONIES/PLATE	% SURVIVAL RELATIVE TO CONTROL
0 (control)*	115.5**	100.00
1.22	143.0	123.81
2.44	115.0	99.57
4.88	132.0	114.29
9.77	134.0	116.02
19.53	145.0	125.54
39.06	116.0	100.43
78.13	140.0	121.21
156.25	127.0	109.96
312.50	125.0	108.23
625.00	147.0	127.27
1250.00	176.0	152.38
2500.00	143.0	123.81
5000.00	54.0	46.75
10,000.00	137.0	118 61

* Solvent Control (100 µl/plate)
** Average of two plates.

RESULTS TABLE 2

A. NAME OR CODE DESIGNATION OF THE TEST COMPOUND: LIQUID POLYMER 11664-97
B. SOLVENT: DMSO
C. TEST INITIATION DATES: 10/22/81 10/27/81
D. TEST COMPLETION DATE: 10/25/81
E. S-9 LOT#: REF050
NOTE: CONCENTRATIONS ARE GIVEN IN MICROGRAMS PER PLATE

TEST	SPECIES	TISSUE	REVERTANTS PER PLATE											
			TA-1535			TA-1537			TA-1538			TA-98			TA-100		
			1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
NONACTIVATION																	
SOLVENT CONTROL	---	---	8	13		7			14	10		26			91	114	
SOLVENT CONTROL	---	---	10	17		8			15	15		20			108	114	
POSITIVE CONTROL••	---	---	0	874		117			230	1525		552			1543	1441	
POSITIVE CONTROL••	---	---	0	632		150			661	1580		845			1515	1374	
TEST COMPOUND																	
1.000000 UG	---	---	14	14		6			10	16		17			24	119	
10.000000 UG	---	---	13	16		5			9	11		23			46	121	
100.000000 UG	---	---	22	21		7			11	11		22			65	117	
500.000000 UG	---	---	30	14		7			13	15		20			88	109	
1000.000000 UG	---	---	32	18		5			14	16		18			103	90	
2500.000000 UG	---	---	17	10		7			2	10		27			80	77	
5000.000000 UG	---	---	2	17		8			1	9		26			106	40	
10000.000000 UG	---	---	1	7		10			0	0		21			46	6	
ACTIVATION																	
SOLVENT CONTROL	RAT	LIVER	11	12		11			25			0	25		112	93	
SOLVENT CONTROL	RAT	LIVER	10	10		16			30			0	23		100	95	
POSITIVE CONTROL•••	RAT	LIVER	45	303		178			862			810	1721		1586	2488	
POSITIVE CONTROL•••	RAT	LIVER	40	345		187			1085			950	1653		1833	2483	
TEST COMPOUND																	
1.000000 UG	RAT	LIVER	6	14		7			16			29	34		75	93	
10.000000 UG	RAT	LIVER	8	12		8			27			48	32		94	110	
100.000000 UG	RAT	LIVER	7	12		9			30			41	24		90	103	
500.000000 UG	RAT	LIVER	13	10		13			38			49	37		130	86	
1000.000000 UG	RAT	LIVER	13	13		7			23			47	27		153	103	
2500.000000 UG	RAT	LIVER	5	5		8			18			28	31		121	105	
5000.000000 UG	RAT	LIVER	7	9		8			20			25	23		91	114	
10000.000000 UG	RAT	LIVER	6	8		8			22			30	25		103	91	

••
TA-1535 SODIUM AZIDE 10 UG/PLATE
TA-1537 9-AMINOACRIDINE 50 UG/PLATE
TA-1538 2-NITROFLUORENE 10 UG/PLATE
TA-98 2-NITROFLUORENE 10 UG/PLATE
TA-100 SODIUM AZIDE 10 UG/PLATE
SOLVENT 100 UL/PLATE

•••
TA-1535 2-ANTHRAPINE 2.5 UG/PLATE
TA-1537 2-ANTHRAPINE 2.5 UG/PLATE
TA-1538 2-ANTHRAPINE 2.5 UG/PLATE
TA-98 2-ANTHRAPINE 2.5 UG/PLATE
TA-100 2-ANTHRAPINE 2.5 UG/PLATE

AMES SALMONELLA/MICROSOME PLATE ASSAY

1. OBJECTIVE

The objective of this study is to evaluate a test material for mutagenic activity in a bacterial assay with and without a mammalian S9 activation system.

2. RATIONALE

The Salmonella typhimurium strains used at LBI are all histidine auxotrophs by virtue of mutations in the histidine operon. When these histidine-dependent cells are grown in a minimal media petri plate containing a trace of histidine, only those cells that revert to histidine independence (his+) are able to form colonies. The trace amount of histidine allows all the plated bacteria to undergo a few divisions; this growth is essential for mutagenesis to occur. The his+ revertants are easily socred as colonies against the slight background growth. The spontaneous mutation frequency of each strain is relatively constant, but when a mutagen is added to the agar the mutation frequency is increased 2- to 100-fold. Cells which grow to form colonies on the minimal media petri plates are therefore assumed to have reverted, either spontaneously or by the action of a test substance to his+ genotype.

3. MATERIALS

A. Indicator Microorganism

The Salmonella typhimurium strains used in this assay were obtained from Dr. Bruce Ames, University of California at Berkeley.[1-5] The following five strains are routinely used:

Strain Designation	Gene Affected	Additional Mutations: Repair	LPS	R Factor	Mutation Type Detected
TA-1535	his G	Δ uvr B	rfa	-	Base-pair substitution
TA-1537	his C	Δ uvr B	rfa	-	Frameshift
TA-1538	his D	Δ uvr B	rfa	-	Frameshift
TA-98	his D	Δ uvr B	rfa	pKM101	Frameshift
TA-100	his G	Δ uvr B	rfa	pKM101	Base-pair substitution

3. MATERIALS (Continued)

The aforementioned strains have, in addition to the mutation in the histidine operon, a mutation (rfa-) that leads to defective lipopolysaccharide coat, a deletion that covers genes involved in the synthesis of vitamin biotin (bio-) and in the repair of ultraviolet (uv) - induced DNA damage (uvrB-). The rfa- mutation makes the strains more permeable to many large molecules. The uvrB-mutation decreased repair of some types of chemically or physically damaged DNA and thereby enhances the strain's sensitivity to some mutagenic agents. The resistant transfer factor plasmid (R factor) pKM101 in TA-98 and TA-100 is believed to cause an increase in error-prone DNA repair that leads to many more mutations for a given dose of most mutagens[5]. In addition, plasmid pKM101 confers resistance to the antibiotic ampicillin, which is a convenient marker to detect the presence of plasmid in the cells.

All indicator strains are kept at 4°C on minimal medium plates supplemented with a trace of biotin and an excess of histidine. The plates with plasmid-carrying strains contain in addition ampicillin (25 µg/ml) to ensure stable maintenance of plasmid pKM101. New stock culture plates are made as often as necessary from frozen master cultures or from single colony reisolates that were checked for their genotypic characteristics (his, rfa, uvrB, bio) and for the presence of plasmid. For each experiment, an inoculum from the stock culture plates is grown overnight at 37°C in nutrient broth (Oxoid CM67).

B. Media

The bacterial strains were cultured in Oxiod Media #2 (nutrient broth). The selective medium was Vogel Bonner Medium E with 2% glucose[7]. The overlay agar consisted of 0.6% purified agar with 0.5 mM histidine, 0.05 mM biotin and 0.1M NaCl according to the methods of Ames et al.[6].

C. Activation System

(1) S9 Homogenate

A 9,000 x g supernatant prepared from Sprague-Dawley adult male rat liver induced by Aroclor 1254 (described by Ames et al.[6]) was purchased from Bionetics Laboratory Products, Litton Bionetics, Inc. and used in this assay.

3. MATERIALS (Continued)

(2) S9 Mix

Components	Concentration Milliliter S9 Mix
NADP (sodium salt)	4 µmoles
D-glucose-3-phosphate	5 µmoles
$MgCl_2$	8 µmoles
KCl	33 µmoles
Sodium phosphate buffer pH 7.4	100 µmoles
Organ homogenate from rat liver (S9 fraction)	100 µliters

4. EXPERIMENTAL DESIGN

A. Dosage Selection

Doses used in the mutagenicity assays were selected from a preliminary toxicity test performed on the strain TA-100. For preliminary toxicity test, 14 doses from 1.0 µg to 10,000 µg per plate for solids and 10 doses from 0.01 µl to 150 µl per plate for liquids were used. In the mutagenicity assays, at least six doses were used with the highest dose exhibiting a 50% toxicity. If the test material is not toxic, 8 doses of 1.0, 10, 100, 500, 1000, 2500, 5000 and 10,000 µg per plate for solids and 0.1, 1, 5, 10, 25, 50, 100 and 150 µl per plate for liquids are used.

If the sponsor specifies doses, no toxicity testing will be performed and the tests are run using the specified doses.

B. Toxicity Studies

To a sterile test tube containing 2.0 ml of overlay agar (placed in a 43°-45°C water bath) the following is added:

- 0.1 ml to 0.2 ml of a solution of the test material to give the appropriate dose.
- 0.2 ml of 10^{-6} dilution of overnight culture.
- 0.5 ml of 0.2M phosphate buffer, pH 7.4.

This mixture is swirled gently and then poured on to nutrient agar plates. After the overlay agar was set, the plates are incubated at 37°C for approximately 24 hours. The number of colonies growing on the plates counted and recorded.

4. EXPERIMENTAL DESIGN (Continued)

C. Mutagenicity Testing

The procedure used is based on the paper published by Ames et al.[6] and is performed as follows:

(1) Nonactivation Assay

To a sterile 13 x 100 mm test tube placed in a 43°C water bath the following is added in order:

(a) 2.00 ml of 0.6% agar containing 0.05 mM histidine and 0.05 mM biotin.

(b) 0.05 ml of a solution of the test chemical to give the appropriate dose.

(c) 0.1 ml - 0.2 ml of indicator organism(s).

(d) 0.50 ml of 0.2M phosphate buffer, pH 7.4.

This mixture is swirled gently and then poured onto minimal agar plates (see 3B, Media). After the top agar has set, the plates are incubated at 37°C for approximately 2 days. The number of his+ revertant colonies growing on the plates is counted and recorded.

(2) Activation Assay

The activation assay is run concurrently with the nonactivation assay. The only difference is the addition of 0.5 ml of S9 mix (see 3C:2, Activation System) to the tubes in place of 0.5 ml of phosphate buffer which is added in nonactivation assays. All other details are similar to the procedure for nonactivation assays.

A detailed flow diagram for the plate incorporation assay is provided in Figure 1.

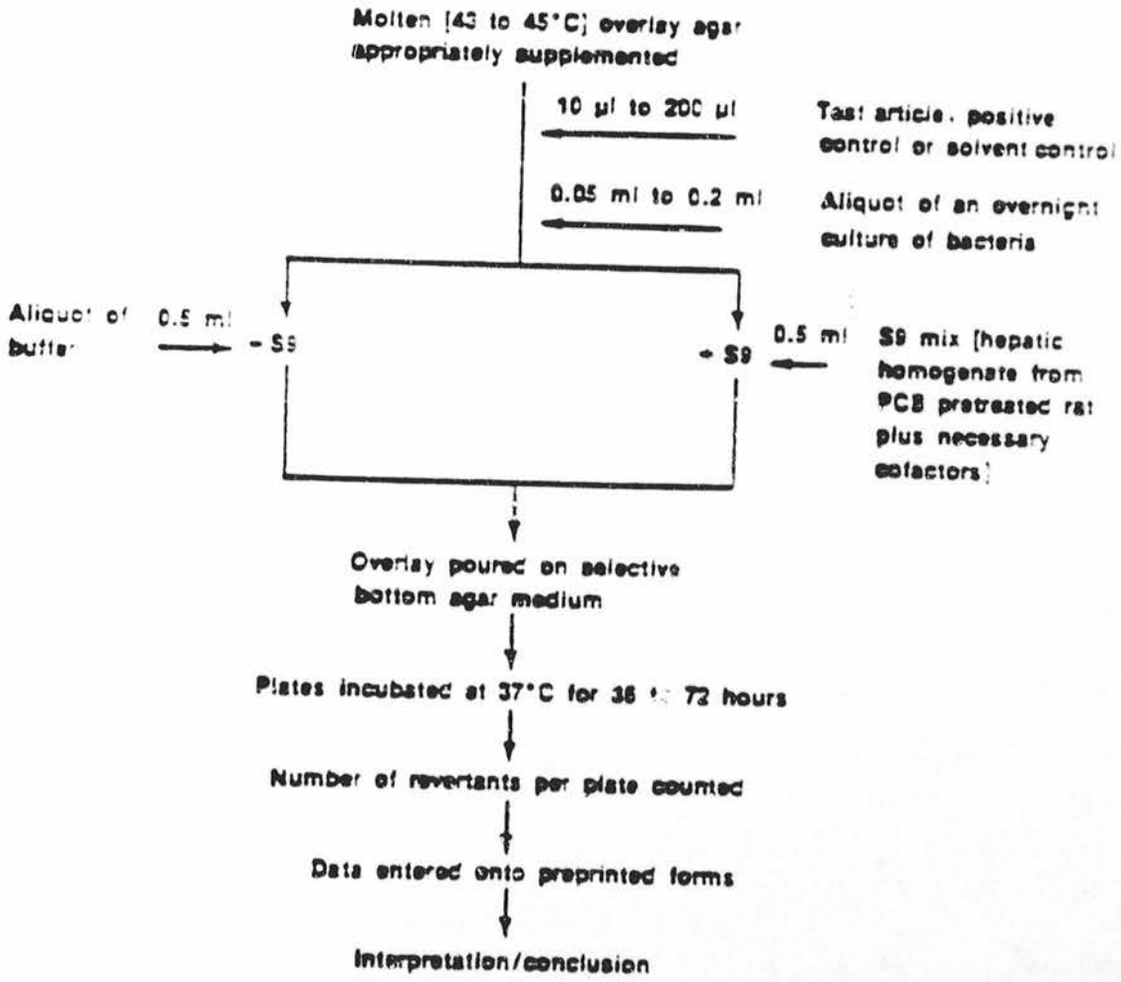
D. Control Compounds

A negative control consisting of the solvent used for the test material is also assayed concurrently with the test material. For negative controls, step 'b' of Nonactivation Assays is replaced by 0.05 ml of the solvent. The negative controls are employed for each indicator strain and are performed in the absence and presence of S9 mix. The solvent used to prepare the stock solution of the test material is given in the Results section of this report. All dilutions of the test material are made using this solvent. The amount to solvent used is equal to the maximum volume used to give appropriate test dose.

Specific positive control compounds known to revert each strain are also used and assayed concurrently with the test material. The concentrations and specificities of these compounds are given in the following table:

FIGURE 1

REVERSE MUTATION ASSAY
(Agar Incorporation Method)



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4. EXPERIMENTAL DESIGN (Continued)

Assay	Chemical	Solvent	Concentration per plate (µg)	Salmonella Strains
Nonactivation	Sodium azide	Water	10.0	TA-1535, TA-100
	2-Nitrofluorene (NF)	Dimethyl-sulfoxide	10.0	TA-1538, TA-98
	9-aminoacridine (9AA)	Ethanol	50.0	TA-1537
Activation	2-anthramine (ANTH)	Dimethyl-sulfoxide	2.5	For all strains

5. EVALUATION CRITERIA

Statistical methods are not currently used, and evaluation is based on the criteria included in this protocol.

Plate test data consist of direct revertant colony counts obtained from a set of selective agar plates seeded with populations of mutant cells suspended in a semisolid overlay. Because the test material and the cells are incubated in the overlay for approximately 2 days and a few cell divisions occur during the incubation period, the test is semiquantitative in nature. Although these features of the assay reduce the quantitation of result, they provide certain advantages not contained in a quantitative suspension test:

- The small number of cell divisions permits potential mutagens to act in replicating DNA, which is often more sensitive than nonreplicating DNA.
- The combined incubation of the test material and the cells in the overlay permits constant exposure of the indicator cells for approximately 2 days.

C. Evaluation Criteria for Ames Assay

Because the procedures used to evaluate the mutagenicity of the test material are semiquantitative, the criteria used to determine positive effects are inherently subjective and are based primarily on a historical data base. Most data sets are evaluated using the following criteria:

(1) Strains TA-1535, TA-1537 and TA-1538

If the solvent control value is within the normal range, a test material producing a positive response equal to three times the solvent control value is considered mutagenic.

5. EVALUATION CRITERIA (Continued)

(2) Strains TA-98 and TA-100

If the solvent control value is within the normal range, a test material producing a positive response equal to twice the solvent control value for TA-98 and TA-100 is considered mutagenic.

The following normal range of revertants for solvent controls are generally considered acceptable:

TA-1535:	8-30
TA-1537:	4-30
TA-1538:	10-35
TA-98 :	20-75
TA-100 :	80-250

(3) Pattern

Because TA-1535 and TA-100 are both derived from the same parental strain (G-46) and because TA-1538 and TA-98 are both derived from the same parental strain (D3052), to some extent there is a built-in redundancy in the microbial assay. In general, the two strains of a set respond to the same mutagen and such a pattern is sought. Generally, if a strain responds to a mutagen in nonactivation tests, it will do so in activation tests. Occassionally, exception to this pattern may also be seen.

B. Dose-Response Phenomena

The demonstration of dose-related increases in mutant counts is an important criterion in establishing mutagenicity. Since, we employ several doses in the actual assay, a dose response would normally be seen with a mutagenic test material. Additional tests may be performed at narrower dose, if the mutagenic test material fails to exhibit a dose-response in the initial assay. However, occassionally it is difficult to generate a dose-response and the test material will be evaluated based on the available data.

C. Reproducibility

If a test material produces a response in a single test which cannot be reproduced in additional runs, the initial positive test data lose significance.

D. Control Tests

Positive and negative control assays are conducted with each experiment and consist of direct-acting mutagens for nonactivation assays and mutagens requiring metabolic biotransformation in activation assays. Negative controls consist of the test material solvent in the overlay agar together with the other essential components. The negative control plate for each

5. EVALUATION CRITERIA (Continued)

strain gives a reference point to which the test data is compared. The positive control assay is conducted to demonstrate that the test systems are functional with known mutagens.

E. Relation Between Mutagenicity and Carcinogenicity

It must be emphasized that the Ames Salmonella/Microsome Plate Assay is not a definitive test for chemical carcinogens. It is recognized, however, that correlative and functional relations have been demonstrated between these two endpoints. The results of comparative test on 300 chemicals by McCann et al.[1] show an extremely good correlation between results of microbial mutagenesis tests and in vivo rodent carcinogenesis assays.

All evaluations and interpretation of the data to be presented in the final report will be based only on the demonstration, or lack, of mutagenic activity.

REFERENCES

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2. Ames, B.N., Gurney, E.G., Miller, J.A. and Bartsch, H.: Carcinogens as frameshift mutagens: Metabolites and derivatives of 2-acetylaminofluorene and other aromatic amine carcinogens. Proc. Nat. Acad. Sci., USA, 69:3128-3132, 1972.

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6. Ames, B.N., McCann, J. and Yamasaki, E.: Methods for detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test. Mutation Res., 31:347-364, 1975.

7. Vogel, H.J. and Bonner, D.M.: Acetylornithinase of E. coli; Partial purification and some properties. J. Biol. Chem., 21897-106, 1956.

Q.A. Inspection Statement
(reference 21 CFR 58.35(b)(7))

PROJECT 2088 LBI Assay No. 599

TYPE of STUDY Ames Plate Test

This final study report was reviewed by the LBI Quality Assurance Unit on 11.18.81. A report of findings was submitted to the Study Director and to Management on 11.18.81.

The short-term nature of this study precluded inspection while it was in process. The Quality Assurance Unit inspects an in-process study of this type approximately once per month to assure that no significant problems exist that are likely to affect the integrity of this type of study.

Marshall L. Ogren
Auditor, Quality Assurance Unit

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